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DIFFERENTIAL METHODS FOR DETECTING THE TYPHOID BACILLUS IN INFECTED WATER AND MILK.*

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It is of the greatest importance in tests of the sanitary quality of water and milk supplies that organisms which specifically cause infectious diseases through such sources shall be subject to methods of rapid and definite isolation. Up to the present time this has been a matter of great difficulty and uncertainty owing to the fact that the disease germs which occur in water and especially in milk are intermingled with, and greatly outnumbered by, other intestinal germs and many species of common so-called air and water bacteria.

CHEMICAL PRECIPITATION AND AGGLUTINATION.

Many methods have been employed in isolating or attempting to isolate the typhoid bacillus from water. Processes involving chemical precipitation have been studied by Vallet,[1] Schüder,[2] Ficker,[3] Müller,[4] Willson,[5] Nietor,[6] and Ditthorn and Gildemeister,[7] the latter using ox bile as an enrichment medium after chemical precipitation.

Agglutination has also been used in enrichment methods for the isolation of *B. typhosus* by Adami and Chopin,[8] Klotz,[9] Shipilewski,[10] and Altschuler.[11]

INDICATOR DYES AND DIFFERENTIAL CHEMICALS.

Methods have been recommended involving the use of dyes as indicators or of chemicals which favor the growth of typhoid germs and retard or exclude other bacteria. In some cases *B. typhosus* has been isolated by the use of a food medium containing both an indicator dye and a differential chemical.

In 1890 Parietti[12] employed a process consisting of the addition of varying amounts of the water to be tested to a series of broth tubes containing increasing quantities of a solution of 4 per cent hydrochloric acid and 5 per cent of carbolic acid. These he incubated at 37° C. and made cultures from the highest acid concentrations in which

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growth occurred. Some years later, Hankin[13] modified this process by selecting the next highest tube in which growth occurred and by starting a new series of tubes from this growth. The higher inoculations of this second series were then finally examined by subcultures.

Elsner[14] in 1896 recommended a medium having 10 per cent of gelatin and 1 per cent of potassium iodide added to an infusion of potato having a reaction of 3.0 per cent normal acid. By means of Elsner's method or by phenolated gelatin several experimenters,[15] [16] were able to isolate the typhoid germ from polluted water.

In 1902, v. Drigalski and Conradi[17] isolated the typhoid bacillus from excreta by nutrose litmus lactose agar containing crystal violet, and Conradi by the use of carbol gelatin plates succeeded in isolating *B. typhosus* from a well at Pecs in Hungary. The Drigalski-Conradi[18] medium is a selective one favoring the growth of *B. typhosus* and *B. coli*. Examinations are made after 24 hours' incubation at 37° C. The colon bacillus produces red non-transparent colonies, while the typhoid colonies are blue or violet and are transparent and of smaller size.

Löffler,[19] [20] and also Lentz and Tietz,[21] [22] have used malachite green agar as a differential medium. Doebert[23] and Nowack[24] have shown that certain varieties of this dye cannot be used, the latter author attributing the difference to the presence of dextrin.

Endo[25] used fuchsin lactose agar decolorized by sodium sulphite and Gähtgens[26] added to this caffeine which had been found by Roth[27] to inhibit the *B. coli*. On this media the *B. coli* is red and the *B. typhosus* colorless. Hoffman and Ficker[28] in 1904 isolated *B. typhosus* from feces and from infected water by using caffeine-nutrose-crystal-violet solution and transplanting by smears on Drigalski-Conradi plates.

The same year Jaksch and Rau[29] isolated typhoid bacteria from the Prague Water Supply and from the Moldau River, and Strószner[30] isolated it from a well near Budapest by the use of caffeine-nutrose-crystal-violet agar.

Methods involving chemical precipitation, agglutination, or even the use of stains have a great tendency to reduce the original number of typhoid bacteria present, and in cases where they are at the start very few in numbers there is a danger that they may be lost sight of altogether. This is also true to a greater or lesser extent with acids, caffeine and other chemical substances used for differentiation.

DIFFERENTIAL FOOD MEDIA.

A simple and valable medium proposed by Hiss[31] and used by the authors does not have this disadvantage and after their enrichment is of importance in the differentiation of *B. typhosus* from *B. coli*. Hiss's medium is semifluid at 37° C. and *B. typhosus* has a tendency to swim out from the colonies forming branchlike processes which distinguish it from *B. coli*. In composition this medium is:

Agar	15 gm.	NaCl	5 gm.
Gelatin	15	Dextrose	10
Liebig's Extract	5	Distilled Water	1,000 c.c.

Reaction 1.0 per cent normal acid.

Other media of this character were originally proposed by Rosenthal[32] in 1895, Klie[33] and Capaldi[34] in 1896, and Hiss in 1897 and 1901. Differentiation with all of these media is based on the high motility of the typhoid bacillus. The differential medium of Hesse which will be described later may be considered to be a refinement of these media which allows of much greater ease in distinguishing between *B. typhosus* and *B. coli.*

NEW METHOD.

The method which the authors desire to propose is not subject to the liability of the loss of *B. typhosus* even when present in very small numbers and has proved to be most successful in the numerous cases in which it has been tried. It is based on a preliminary cultivation of the sample of water, milk, or feces to be tested in lactose bile (34, 35, 36) as an enrichment medium and plating from this on Hesse agar (37, 38) to get the characteristic typhoid colonies.

Examination of typhoid feces.—Hesse agar may be used directly in the examination of the feces of suspected cases of typhoid fever, but the authors have found in cases where the test was uncertain, due to the outnumbering of the *B. typhosus* by the *B. coli*, that preliminary cultivation in lactose bile insures definite results. The mode of procedure is to plant dilutions of the feces in Hesse agar and at the same time inoculate with the feces a fermentation tube containing lactose bile, both being incubated at 37° C.

If on the second day the cultures from the Hesse agar are uncertain a series of dilutions are made from the lactose-bile tube which, if necessary, may be followed by a third series on the following day from the two-day-old culture in the fermentation tube.

The preliminary cultivation in the lactose-bile solution greatly increases the number of the *B. typhosus* present so as to render its identification more definite. It often gives more characteristic colonies where there is uncertainty in identification and at the same time it preserves the cultures for future transplantations when second or third tests are necessary.

Lactose-bile medium.—This medium which was first proposed by one of the authors has now come into general use in testing for *B. coli* in water, and is also used in some laboratories in testing for the amount and extent of fecal contamination in milk. It consists of sterilized, undiluted fresh ox gall (or an 11 per cent solution of dry fresh ox gall) to which has been added 1 per cent of peptone and 1 per cent of lactose. Fermentation tubes holding 40 c.c. of the sterilized liquid are inoculated with varying amounts up to 10 c.c. of the water or milk to be tested. After 48 hours of incubation at 37° C. at least 25 per cent of the length of the fermentation tube will be filled with gas if *B. coli* is present. In attenuated cultures a three-day test is sometimes required.

The authors have found that *B. typhosus* grows even more abundantly in this media than does *B. coli*, that other organisms are rapidly overgrown, and that *B. coli* itself may be finally overgrown by *B. typhosus*. But as *B. typhosus* does not produce a gas and does not in any other way indicate its presence by the appearance of the medium, it was necessary to go through a very laborious process in order to isolate the organism. While experiments of this character were under consideration, the discovery of a new medium by Dr. W. Hesse was announced.

Hesse agar.—This medium easily distinguishes between *B. typhosus* and *B. coli*, and unlike other distinguishing media has no tendency to reduce the number of typhoid bacilli present. By its use the authors were able to isolate *B. typhosus* from preliminary cultivations in lactose bile of samples of suspected water supplies and artificially infected milks. In such cases the preliminary cultivation in bile is necessary in order to increase the typhoid germs in numbers over the various other species always present in contaminated supplies.

The Hesse medium contains less agar and more salt than standard agar and is made from Liebig's extract of beef instead of the pure meat juice. Its composition is as follows:

Agar	5 gm. (4½ gm. absolutely dry)
Peptone, Witte	10 "
Liebig's Extract of Beef	5 "
Salt	8½ "
Distilled Water	1,000 c.c.

In a study of this medium the authors have found:

That the agar must be previously dried for half an hour at 105° C. as it sometimes contains as high as 20 per cent of moisture and an exact amount of agar is highly important in order that the medium may be very soft but still retain its position on the plate. With agar dried as described the proper amount to use is 4½ gm. per liter. This is probably equivalent to that used by Hesse, as according to description our agar was more thoroughly dried. A small sample of the agar may be dried and the moisture allowed for in making the medium.

That the medium must be stored in an ice chest the air of which is kept saturated with moisture, and the cultures must be incubated in a 37° C. incubator also with moisture-saturated air so that the medium will not change its consistency. It is a decided improvement to use porous earthenware tops[42] to the petri dishes instead of the ordinary glass tops in order to prevent spreading and to keep the colonies in their natural and characteristic condition.

PREPARATION OF HESSE AGAR.

The preparation of the medium is in detail as follows: Four and one-half grams of dry agar is dissolved in 500 c.c. of distilled water by heating over a free flame, making up loss in weight by evaporation. Into another vessel 500 c.c. of distilled water is poured and to this is added 10 gm. of peptone, 5 gm. of Liebig's meat extract, and 8.5 gm. of salt. This is heated until all is dissolved and the loss in weight by evaporation is made up by adding distilled water.

Add the two solutions together; boil 30 minutes; make up loss in weight with distilled water, filter through absorbent cotton held in the funnel by cotton flannel, passing the filtrate through several times until perfectly clear. Test the reaction and adjust, if necessary, to 1.0 per cent normal acid, and tube, using 10 c.c. of medium in each tube. Sterilize for twenty minutes at 120° C. (15 pounds pressure) in an autoclave. Cool with running tap water and store in an ice chest, the air of which is saturated with moisture.

Extensive experiments have been carried on to see if any improvement could be made in the contents of this medium. Different amounts of agar were tried, from 4 to 7 gm. being used taking each half-gram in between these two points. Standard extract of fresh beef was tried but did not seem to give as good results for the purpose as Liebig's extract. Varying amounts of salt from none to 10 gm. with standard broth and also with Liebig's extract were used but the amount given in the formula was found to give the most characteristic results.

The preparation of dilution plates is carried out as follows:

Eight tubes containing each 9 c.c. of sterilized distilled water are set in a rack together with a series of eight sterilized petri dishes and both numbered from 1 to 8. Dr. Hesse uses physiological-salt solution for dilution but our experiments show that distilled water always gives equally good results.

Into Tube 1 place 1 gm. or 1 c.c. of feces, or 1 c.c. of bile solution containing the feces, water, or milk to be tested and which has been previously inoculated and incubated at least 24 hours at 37° C. After thoroughly mixing with the 9 c.c. of distilled water in the tube place 1 c.c. of this mixture into Plate 1, and 1 c.c. into Tube 2. From Tube 2 place 1 c.c. into Plate 2 and 1 c.c. into Tube 3. Proceed in this manner through the series. Now add to each petri dish 10 c.c. of the liquefied Hesse agar cooled to 40° C. and mix thoroughly. First cool in the ice chest to set the medium and incubator at 37° C. for 24 hours.

The use of physiological-salt solution is unnecessary in the dilution work for the various plates used, but it should be used in the dilution of the anti-typhoid serum, described later. The following table shows that distilled water may be used with equal success in the dilution work previous to plating Hesse agar.

Table 1 shows the use of physiological-salt solution compared with sterilized distilled water for dilution work using three strains of *B. typhosus*:

TABLE 1.

DILUTION	BACTERIA PER C. C.				
	Plate 1 1–10	Plate 2 1–100	Plate 3 1–1,000	Plate 4 1–10,000	Plate 5 1–100,000
Strain No. 1					
NaCl sol.	1.200	116*	17*	2*	0
Sterile water	1,290	97*	15*	2*	0
Strain No. 2					
NaCl sol.	2,160	227	24*	3*	0
Sterile water	2,140	229	24*	2*	0
Strain No. 3					
NaCl sol.	13,460	1,760	184*	19*	2*
Sterile water	13,400	1,740	185*	21*	2*

Plates marked with a star gave very characteristic colonies on Hesse agar but where there was a large number of bacteria present the results were not characteristic.

Strain No. 1 represented an old stock culture of *B. typhosus* from which very characteristic colonies could not be obtained on the medium until after rejuvenation with lactose bile for 12 hours.

Strain No. 2 was a culture obtained from Dr. Billings of the City of N. Y. Board of Health.

Strain No. 3 was a culture from Bellevue Hospital.

When feces are plated directly on Hesse medium, the amount taken and the dilution being known, the number of each characteristic species of bacteria is estimated. When feces are inoculated into bile solution the presence only and not the number of *B. typhosus* is determined.

When milk or water are incubated with bile solution varying dilutions in bile tubes may be made to obtain the approximate number of *B. typhosus* present but in practice the presence of the germ is all that it is necessary to determine.

CHARACTERISTICS OF *B. TYPHOSUS.*

B. typhosus is characteristic on Hesse agar only when the dilution is sufficiently high to produce but a few bacteria on the plate. It is distinguished from *B. coli* by the formation of colonies of much larger size, often several centimeters in diameter and consisting of a broad translucent or scarcely turbid zone between the white opaque center or nucleus and the perfectly circular narrow white seam or edge. These characteristics are shown in the accompanying figure giving a dilution of *B. coli* and *B. typhosus* taken by the authors from the Hudson River at Hastings. The culture was previously grown in lactose-bile solution.

The second illustration is a pure culture of *B. typhosus* taken from a stream and pond used as a local private water supply which has been the source of a number of cases of typhoid fever.

These and the other pure cultures of typhoid bacilli isolated were all found to give the proper results when tested by agar and gelatin plates, stabs and slopes, indol reaction, milk, litmus, lactose agar, dextrose broth, lactose bile, potato, Hiss medium, Drigalski-Conradi medium, Hesse agar, morphology, motility, pathogenesis, Pfeiffer reaction, absorption test and Widal reaction [43] [44] [45] [46] (1–1,000). Negative results were obtained on gas, and indol production and positive or characteristic results were obtained by the other tests. Animal subcutaneous inoculations with moderate quantities of 24-hour broth cultures gave negative results. Large quantities produced toxemia.

Additional pure cultures of *B. typhosus* used in the comparative tests were kindly supplied by Dr. Benjamin White of the Hoagland Laboratory, Brooklyn, N. Y., and were from the following sources:

B. typhosus K, isolated by Kruse, Bonn, June 1907, from feces.
B. typhosus L–1, isolated at Lister Institute, 1907, from feces.
B. typhosus L–2, isolated at Lister Institute, 1907, from feces.
B. typhosus L–6, isolated at Lister Institute, 1907, from feces.
B. typhosus M, isolated by Trommsdorf, Munich, from feces.
B. typhosus N, isolated by Neisser, Frankfort, from feces.
B. typhosus P, isolated by Besredka, Pasteur Institute, Paris, from feces.
B. typhosus H, isolated by Neumann, Heidelberg, June, 1907, from feces.

B. paratyphosus, *B. pyocyaneus*, *B. fluorescens liquefaciens*, and some forms of very motile *B. coli* sometimes give a similar appearance. They may be readily distinguished by the absence of a transparent zone and by the Widal reaction. The fermentation test or the planting in the various differential media may be necessary in case of indecision.

The Widal reaction may be made directly from the colonies on the Hesse plate as well as by first planting in beef broth for a period of from 12 to 24 hours.

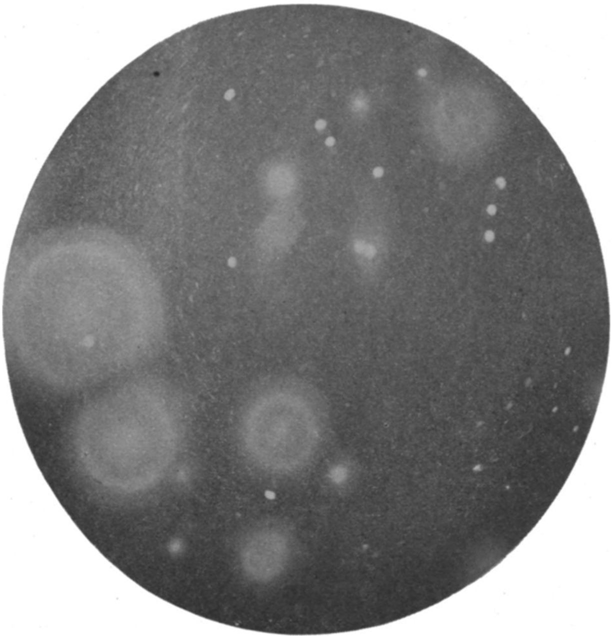

FIG. 1.—*B. coli* (small colonies) and *B. typhosus* (large colonies) taken from the Hudson River and transplanted from lactose bile to Hesse agar.

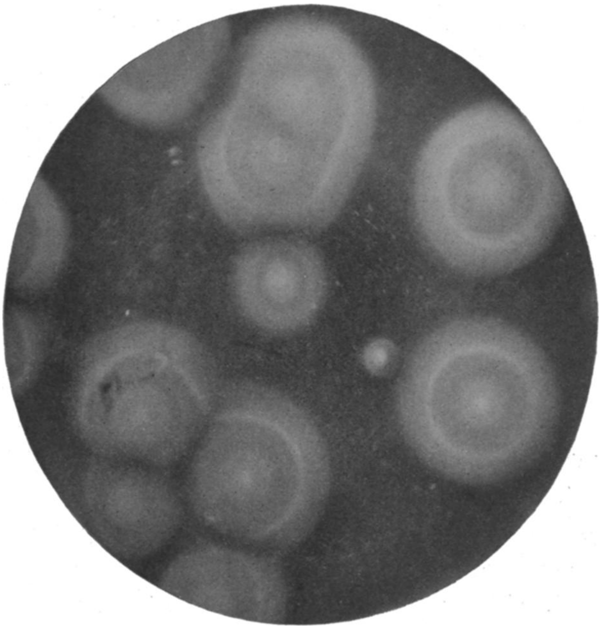

FIG. 2.—Pure culture of *B. typhosus* taken from a stream and pond at Hastings, N. Y., used as a private water supply and the source of several cases of typhoid fever.

THE WIDAL TEST.

To make the Widal test take up a small portion of the culture in a capillary glass tube, place a drop on a cover glass, and invert it over a rubber ring on a glass slide, using vaseline on the edges so as to prevent evaporation and consequent movement by currents in the drop.

If the bacteria are motile rods resembling *B. typhosus*, add a drop of equal size of highly diluted anti-typhoid serum. If in the course of a few minutes the bacteria cease their motions and agglutinate, the presence of *B. typhosus* is practically established, but it is well to make the other cultural and biochemical tests to see that they correspond to the reactions of the typhoid bacillus. In rare cases some strains of *B. coli* and allied species may give the Widal test, but almost invariably in dilutions of 1–50 or less. The high-power dry lens is best for this work.

To determine the point of highest dilution at which agglutination takes place the Widal test is best made in tubes and examined macroscopically. Place a series of small test tubes in a rack and pour into each varying dilutions of anti-typhoid serum. To each tube add an equal amount of 24-hour broth culture of *B. typhosus* and incubate at 37° C. for at least three hours. The highest point at which precipitation takes place is the highest point of agglutination.

CONCLUSIONS.

Bile is a natural medium for the growth of the typhoid bacillus and it retards the growth of other bacteria except *B. coli.* When lactose is present in the bile *B. coli* causes copious gas formation and soon produces sufficient acidity to retard its own growth so that the final result is a predominance of *B. typhosus* which has been multiplying rapidly in the meantime and is not materially affected by this acidity.

When samples of water or milk are inoculated into lactose bile and incubated at 37° C. and then transplanted in varying dilutions into Hesse agar, very characteristic colonies are formed in the high dilutions when the typhoid bacillus is present.

By means of the method described we have been able to isolate typhoid cultures from feces with certainty at any stage of the disease. We have isolated it from milk artificially infected with small numbers of *B. typhosus*. We have isolated it from the Grass River used as a source of water supply for Canton, N. Y., from a pond and stream used as a local private water supply at Hastings, N. Y., and from two points in the Hudson River.

At Canton and Hastings it was obtained from bile tubes containing 10 c.c. of the water, and in the Hudson River near Hastings, at the time of the local typhoid fever epidemic at that point, it was obtained in bile tubes containing only 1 c.c. of the water.

Any volume of water may be tested provided at least four times as much lactose bile as water is added to the bottle before incubation. In routine water analysis when gas is formed in the bile tube, cultures should be plated out on Hesse media to determine the presence of *B. typhosus*. If characteristic colonies are obtained they should be examined for morphology and motility under the microscope and the Widal test applied. This practically demonstrates their presence but they should then be isolated in pure culture and plated on the various distinguishing media as confirmative tests.

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